Instructions for use

BCR-ABL1 Screening dPCR OncoKit

Ref. IMG-424

RUO

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For any questions about the applications of this product or the protocols thereof, please contact our Technical Department:

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Modifications to the instructions for use (IFU)		
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01 General information

The Philadelphia (Ph) chromosome is the result of an abnormality in chromosome 22 caused by a reciprocal translocation in which a portion of chromosome 9 becomes attached to chromosome 22, forming the *BCR-ABL1* oncogene. The Philadelphia chromosome is found in the bone marrow cells of most people with chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). It encodes the BCR-ABL1 protein, a type of tyrosine kinase that causes certain cells (white blood cells) to grow and divide out of control in diseases such as CML and ALL.

The genes involved in the formation of the *BCR-ABL1* oncogene are the *BCR* gene at locus *22q11* (chromosome 22) and the *ABL1* gene at locus *9q34* (chromosome 9). Different forms of the *BCR-ABL1* chimeric oncogene can be identified based on breakpoints in the *ABL1* and *BCR* genes:

- Major (M-BCR) or p210: e13a2, e13a3, e14a2 or e14a3.
- Minor (m-BCR) or p190: e1a2 or e1a3.
- Micro (µ-BCR) or p230: e19a2 or e19a3.

The most prevalent form is M-BCR (major breakpoint cluster region), found in 95% of the transcripts. This is followed by the minor (m-BCR) and micro (µ-BCR) forms, present in 70% and 25% of the transcripts, respectively. Atypical transcripts have also been identified, where the breakpoint is located between intron 1 of *ABL* and either intron 6 or 19 of *BCR*, among others, but they are very rare. Different *BCR-ABL* have been developed, which are important for diagnosing or ruling out CML or ALL.

References

- *Burmeister T, Reinhardt R. A multiplex PCR for improved detection of typical and atypical BCR-ABL fusion transcripts. Leuk Rese. 2008 Apr; 32(4): 579-85 doi: 10.1016/j.leukres.2007.08.017. Epub 2007 Oct 24. PMID: 17928051.*
- *Cross NC, White HE, Colomer D et al. Laboratory recommendations for scoring deep molecular responses following treatment for chronic myeloid leukemia. Leukemia 2015 May;29(5):999-1003. doi: 10.1038/leu.2015.29. Epub 2015 Feb 5. PMID: 25652737; PMCID: PMC4430701.*
- *Maier J, Lange T, Cross M, et al. Optimized Digital Droplet PCR for BCR-ABL. J Mol Diagn. 2019 Jan;21(1):27-37. doi: 10.1016/j.jmoldx.2018.08.012. Epub 2018 Oct 19. PMID: 30347270.*

02 Intended use

BCR-ABL1 Screening dPCR OncoKit is a screening test for the detection and quantification of *BCR-ABL1* and *ABL1* messenger RNA (mRNA) transcripts from peripheral blood complementary DNA (cDNA) samples in patients diagnosed with or suspected of having t(9;22)-positive chronic myeloid leukemia (CML).

The kit employs a combination of oligonucleotides and fluorescent hydrolysis probes in a multiplex assay that allows to differentiate between M-BCR, m-BCR, and µ-BCR breakpoints and to quantify them in relation to the *ABL1* gene using digital PCR (dPCR) technology. The subtypes that can be detected for each of the major breakpoints are:

- Major (M-BCR) or p210: e13a2, e13a3, e14a2 and/or e14a3.
- Minor (m-BCR) or p190: e1a2 or e1a3.
- Micro (µ-BCR) or p230: e19a2 or e19a3.

BCR-ABL1 Screening dPCR OncoKit does not allow detection of rare *BCR-ABL1* splice variants such as p195 or e6a2, p200 or e8a2 and p225 or e18a2.

BCR-ABL1 Screening dPCR OncoKit is for research use only and is strictly intended for technical healthcare and molecular biology professionals who need to identify *BCR-ABL1* fusions.

03 Technical characteristics

BCR-ABL1 Screening dPCR OncoKit is designed to analyze cDNA obtained after reverse transcription of RNA extracted from peripheral blood.

Separate cDNA probes were designed for the validation of BCR-ABL1 Screening dPCR OncoKit for each of the Major (M-BCR), minor (m-BCR), and micro (µ-BCR) breakpoint cluster regions, along with a region of the *ABL1 wild-type* gene (endogenous). Detection and quantification of the fusion transcript and the endogenous gene allows to calculate the frequency of the fusion in the sample. Additionally, positive cDNA samples from previously genotyped controls were analyzed.

Comprehensive validation allows to establish the following **technical specifications**:

- **Type of sample**: cDNA from peripheral blood.
- **Positive control**: Synthetic DNA fragments with M-BCR, m-BCR and µ-BCR and a region of *ABL1* as an endogenous control.
- **Limit of detection**: 0.1%
- **Number of reactions per sample**: 1
- **Number of targets**: (M-BCR, m-BCR and µ-BCR breakpoint cluster regions and a region of *ABL1* as an endogenous control).
- **dPCR program duration**: 01:45

BCR-ABL1 Screening dPCR OncoKit is compatible with the *QuantStudio™ Absolute Q™ Digital PCR System* (Thermo Fisher Scientific) with FAM™, VIC™ and ABY™ fluorescence channels. The device uses *QuantStudio Absolute Q Digital PCR System Software* for semi-automated result analysis.

04 Conditions for sample collection, handling and preparation

Sample collection, transport, storage and preparation are critical steps for performing assays for mRNA transcript detection and quantification. Some of the most important requirements for the handling of these samples are highlighted below. The stability of the samples under transport and storage conditions other than those listed below has not been evaluated with BCR-ABL1 Screening dPCR OncoKit.

- **Sample collection and storage.**

  For optimal results, whole blood samples should be collected in *PAXgene* tubes intended for intracellular RNA purification from whole blood, following the manufacturer's guidelines. If RNA extraction is not performed immediately after collection, it is recommended to store the samples at 4°C for up to 5 days without separating the plasma from the cells. For longer storage, it is recommended to freeze the specimens at -20°C to -70°C.

- **Sample transport:**

  If mRNA extraction is performed in a laboratory other than the one where the sample was collected, it is recommended to ship the sample within 5 days of collection, at 4°C and on dry ice.

- **RNA extraction, working conditions and storage:**

  Use a suitable manual or automated human RNA extraction method. The yield and purity of extracted RNA may differ depending on the extraction method. The *Maxwell® 16 LEV simplyRNA Tissue Kit* (Promega, ref: AS1280) with the corresponding *Maxwell® 16 Instrument* (Promega) has been successfully used for automated extraction.

  Work surfaces and equipment should be thoroughly cleaned with a special RNAse decontamination solution prior to RNA extraction, and all consumables should be RNase-free.

  mRNA should be kept at a temperature of 4°C for immediate use. For short-term and long-term use, it is recommended to keep samples frozen at -20°C and -80°C, respectively. Repeated freezing and thawing of RNA is discouraged as it may affect its integrity.

- **Reverse transcription (RT-PCR) and storage of cDNA:**

  Reverse transcription of mRNA to cDNA was performed with the *SuperScript™ VILO™ cDNA Synthesis Kit* (Thermo Fisher Scientific) using two-step RT-PCR and 1,000 ng mRNA. Using lower amounts of starting material may adversely affect the detection of *BCR-ABL1* transcripts that are underrepresented in the patient's blood.

  The cRNA should be kept at a temperature of 4°C for immediate use. For short-term and long-term use, it is recommended to keep samples frozen at -20°C and -80°C, respectively.

05 Warnings and precautions

- We recommend strictly following the instructions in this manual, especially regarding the handling and storage conditions for the reagents.
- RNAase/DNase contamination can lead to the degradation of RNA samples and cDNA products. Use pipette tips with filters and nuclease-free tubes. Thoroughly clean all work surfaces before commencing work.
- The diagnostic efficacy of this test has only been established for blood collected in *PAXgene* tubes. The performance of this test has not been evaluated with other specimen types or collection methods.
- The reliability of the results depends on the correct performance of specimen collection, transport, storage and processing as outlined in section "04. Conditions for sample collection, handling and preparation". Other sample handling issues may lead to inaccurate results.
- Samples with target transcript concentrations below the established limit of detection may lead to erroneous results.
- Risk prevention and good laboratory practice.
- Any skin conditions, cuts, abrasions, and other skin lesions should be properly covered or protected.
- Do not pour reagent residues into the drinking water system. It is recommended to use the waste containers set out by the legal regulations and to manage them via an authorized waste manager.
- In the case of accidental spillage of any of the reagents, avoid contact with skin, eyes and mucous membranes and wash with plenty of water.
- Product Material Safety Data Sheets (MSDS) are available on https://healthincode.com/kits-de-analisis/.
- This product requires the handling of samples and materials of human origin. It is recommended to consider all materials of human origin as potentially infectious and to handle them in accordance with the OSHA Biosafety Level 2 standard for bloodborne pathogens. Alternatively, other relevant biosafety practices should be used for materials that contain or may contain infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic or corrosive and do not cause biological environmental contamination.

- This kit has been validated with specific equipment and under specific conditions that may vary significantly in other laboratories. It is therefore recommended that each laboratory perform an internal validation when using the kit for the first time.
- The manufacturer is not responsible for the assay failing to work properly when the reagents included in the kit are replaced by other reagents not supplied by Health in Code, S.L.
- The manufacturer does not guarantee the reproducibility of the assay when the user includes reagents not validated by Health in Code, S.L., considering them equivalent to those supplied in the kit.

06 Kit contents and storage conditions

The BCR-ABL1 Screening dPCR OncoKit is composed of the following reagents:

- **BCR-ABL1 Screening Master Mix**, which contains:
  - specific oligonucleotides.
  - probes made by Applied Biosystems™ labelled with FAM™, VIC™, JUN™ and ABY™ for the detection of BCR-ABL1 fusion transcripts (M-BCR, m-BCR and µ-BCR) and *wild-type ABL1* gene, respectively.
  - nuclease-free water.
- **Positive control**: contains synthetic DNA fragments with the 3 *BCR-ABL1* fusion transcripts at a concentration of 33% relative to the *ABL1* gene.

The kit contains enough lyophilized reagents for 24 digital PCR reactions. Physical characteristics, quantities and storage conditions are shown in Table 1.

Table 1. Components of the BCR-ABL1 Screening dPCR OncoKit.

Reagents	Support color	Quantity	Storage
BCR-ABL1 Screening Master Mix	Green pad	2 X 12 rxn	4°C
Positive control	Green cap	6 rxn	4°C

07 Equipment, reagents and materials not supplied

Equipment:

- 10 µL, 20 µL and 200 µL micropipettes
- Vortex
- Centrifuge
- *QuantStudio Absolute Q Digital PCR* (Thermo Fisher Scientific)

Reagents:

- Nuclease-free water
- Absolute ethanol
- *Absolute Q DNA Digital PCR Master Mix (5X)* (Ref. A52490)
- *Isolation Buffer* (Ref. A52730)
- *SuperScript™ VILO™ cDNA Synthesis Kit* (Thermo Fisher Scientific)

Materials:

- Pipette tips with filter (10 µL, 20 µL and 200 µL)
- 1.5 mL sterile tubes
- Powder-free latex gloves
- *MAP16 Plate Kit* (Ref. A52865)

Complementary kits

For the investigation of hematological disorders, Health in Code, S.L. also offers the following real-time PCR kits for specific targets:

- Imegen® BCR ABL1 Screening (ref. IMG-108)
- Imegen® m-BCR ABL (ref. IMG-121)
- Imegen® M-BCR ABL (ref. IMG-122)
- Imegen® Inv16 (ref. IMG-109)
- Imegen® PML-RARA Screening (ref. IMG-130)
- Imegen® PML-RARA (ref. IMG-111)
- Imegen® NPM1 (ref. IMG-235)
- Imegen® MPL (ref. IMG-236)

By fragment analysis:

- Imegen® FLT3 (ref. IMG-238)
- Imegen® CALR (ref. IMG-237)

By digital PCR (dPCR) analysis:

- FLT3-TKD2 dPCR OncoKit (ref. IMG-405)
- cKIT dPCR OncoKit (ref. IMG-407)

By Next-Generation Sequencing (NGS):

- Haematology OncoKitDx (ref. IMG-363)

08 Assay protocol

08.1 | Preparation of reagents

The first step before starting the protocol is to rehydrate the dried reagents:

- *BCR-ABL1 Screening Master Mix*: add 20 µL of nuclease-free water to each vial.
- **Positive control**: add 50 µL of nuclease-free water to the vial.

NOTE: It is recommended to store the rehydrated reagents frozen at – 20°C.

08.2 | Preparation of amplification reactions

In order to estimate the quantity of reagents required, the number of samples and controls to be analyzed simultaneously must be taken into account. We recommend adding one more reaction or increasing the volume of each reagent by 10% when making the calculations (item 4 of the protocol).

In each assay, it is recommended to include the positive control provided in the kit and a negative PCR control to rule out contamination of the reagents.

The protocol for the preparation of amplification reactions is specified below:

01 Thaw the cDNA samples.

NOTE:

> Take into account that prior dilution of the cDNA may be required, as indicated in section "10. Troubleshooting".

> Samples of suboptimal quality should be centrifuged at 10,000 x g or at the maximum available speed for 1 minute; the sample should then be drawn from the top of the tube.

02 Thaw all the reagents in the kit, the nuclease-free water and the *Absolute Q DNA Digital PCR Master Mix* (5X) (not included in the kit). It is important to allow all the components to reach room temperature before use.

NOTE: Keep all the reagents on ice and protected from light when not in use.

03 Vortex all the reagents, including *Absolute Q DNA Digital PCR Master Mix* (5X), for 10 seconds.

04 Prepare the PCR mix in a 1.5 mL tube with the reagents listed below.

Reagent	Volume per reaction**
BCR-ABL1 Screening Master Mix	1.5 µL
Absolute Q DNA Digital PCR Master Mix (5x)*	2 µL

(*) Reagent not supplied by the manufacturer (see section "07. Equipment, reagents and materials not supplied")

(**) Adjust the final volume according to the number of reactions as described at the beginning of the section.

05 Vortex the PCR mix for 10 seconds and then spin for 30 seconds. Aliquot 3.5 µL into different PCR tubes.

06 Transfer 6.5 µL of the supernatant from the samples into each PCR tube containing the reaction mix prepared in step 5. For the negative control, add nuclease-free water.

07 Use of the following options to add the mix from item 6:

- Pipette up and down 10–20 times, taking care not to create bubbles.
- Vortex 3–5 times for 1 second at a time.

08 Centrifuge for 1 min at 10,000 x g.

09 Hold the pipette at a 45° angle and load 9 µL of the PCR mix into the bottom of each well of the MAP16 microfluidic array plate, depressing until the first pipette stop.

10 Then, holding the pipette tip against the side of the well at a 45° angle, dispense 15 µL of Isolation Buffer on top the reagents to prevent mixing and bubble formation.

Optional: wait 15 min before pressing Start and keep the plate inside the device in case bubbles have formed or the reagents have mixed during loading.

08.3 | Real-time PCR program setup

Load the microfluidic array plate (MAP16)

Mount the strips for the MAP plates in the 4 well columns. Make sure the columns are completely covered and place the MAP plate caps on all the columns, including unused ones. Download the user guide: *MAN0025621 QuantStudio™ Absolute Q™ Digital PCR System User Guide* from www.thermofisher.com, and follow the instructions provided in Section 2.

PCR program setup

Place the MAP plate in the *QuantStudio™ Absolute Q™ System*, adjust the optimal channels and PCR settings as specified in Table 2 and set up the dPCR program as described in Table 3.

- Experiment setup:

Table 2. Channel setting for fluorophore detection on the QuantStudio™ Absolute Q™ dPCR System. QC: quality control.

Fluorophore	Molecular target	Analysis
FAM™	M-BCR-ABL1	signal
VIC™	m-BCR-ABL1	signal
JUN™	μ-BCR-ABL1	signal
ABY™	ABL1	signal
ROX™	QC	QC

◊ Optimal PCR program:

Table 3. Optimal PCR program for QuantStudio™ Absolute Q™ dPCR (Thermo Fisher Scientific).

Stage	No. of cycles	Temperature	Time
Initial denaturation	1	96°C	10 minutes
PCR Denaturation, annealing and extension	40	96°C	5 seconds
		62°C	15 seconds

↘ Array reading and obtaining results

Following completion of the PCR, follow the instructions in the user guide for the *QuantStudio™ Absolute Q™* digital PCR system available from www.thermofisher.com.

09 Analysis of results

The approach used in the design of BCR-ABL1 Screening dPCR OncoKit is based on a non-competitive multiplex dPCR assay in which *BCR-ABL1* transcripts are detected with different fluorophores, as shown in Table 4.

Table 4. Information on the targets for each probe used in BCR-ABL1 Screening dPCR OncoKit

System	Fluorophore	Function
Screening BCR-ABL1	FAM™	The FAM™ probe identifies the M-BCR (p210) transcript
	VIC™	The VIC™ probe identifies the M-BCR (p190) transcript
	JUN™	The JUN™ probe identifies the µ-BCR (p230) transcript
	ABY™	The ABY™ probe binds to binds to the wild-type *ABL1* gene

Sample analysis is accomplished by the specific software of the PCR thermocycler used: *QuantStudio™ Absolute Q™ Digital PCR Software*. To determine whether a sample is positive and for which fusion transcript, follow the steps below.

01 Go to *"ANALYSIS"* section to visualize and select a sample.

02 At the bottom of the screen, choose the channel you want to visualize (FAM™, VIC™ or JUN™ for the fusion transcripts, ABY™ for *ABL1*).

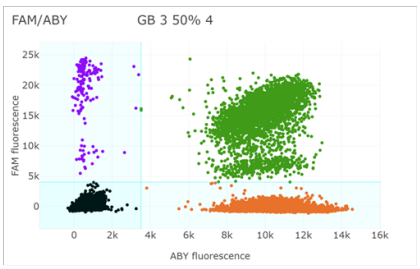
03 Select option to display the data in a two-dimensional (2D) or scatter plot. After adjusting the X and Y axes in the 2D plots, up to four clusters may be identified (Figure 1):

- The cluster of black data points represents the sections where there has been no amplification and, therefore, no signal has been recorded for any of the probes.
- The cluster of orange data points corresponds to areas where only the ABY™, which hybridizes with a region of the *ABL1* gene serving as the endogenous control, has produced positive signals.
- The cluster of purple data points corresponds to sections positive for aberrant BCR-ABL1 transcripts detected using the corresponding channel (FAM™, VIC™ and JUN™).
- The cluster of green data points, or combined group, corresponds to positive sections where signals were detected for both the mutated transcript and the wild type *ABL1* gene.

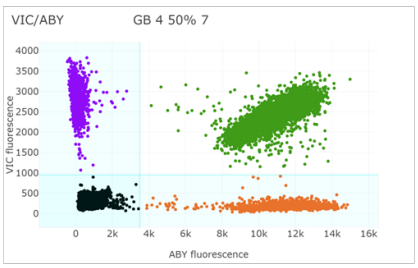
Figure 1 shows the possible results for the major, minor and micro isoforms of *BCR-ABL1* and the positive control included in the kit.

The BCR-ABL1 Screening dPCR OncoKit also allows for the distinction between M-BCR translocation subtypes (Figure 2). A sample containing the e14a2 or e14a3 fusion transcripts results in a 2D plot with two purple clusters and two green clusters, similar to the positive control in the kit (Figure 1A). However, a sample containing the e13a2 or e13a3 fusion transcripts results in a single green and purple cluster in the 2D plot.

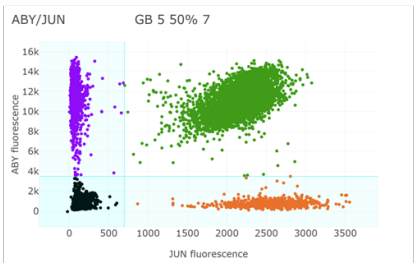
A) Detection of M-BCR (p210). Channel FAM™/ABY™



B) Detection of m-BCR (p190). Channel VIC™/ABY™



C) Detection of µ-BCR (p230). Channel ABY™/JUN™



D) Detection of ABL1 wild-type. Channel FAM™/ABY™

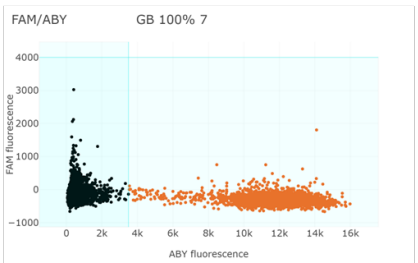
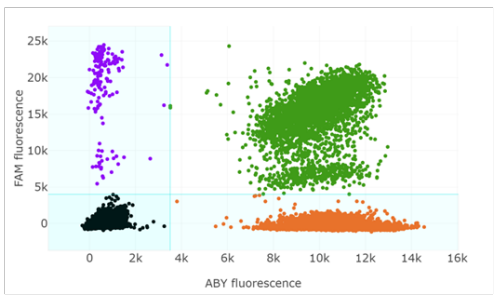


Figure 1. Two-dimensional (2D) plots for quantification of BCR-ABL1 versus ABL1 transcripts with the BCR-ABL1 Screening dPCR OncoKit. ***A-C)*** *Positive control. The plots observed are obtained by selecting the specific fluorescence channel for each transcript (FAM™, VIC™ o JUN™) relative to ABL1 (ABY™).* ***D)*** *Negative control. No amplification is observed for the specific targets of the BCR-ABL1 transcripts. In all cases, the cluster of black data points represents sections where no amplification has occurred.*

A) Detection of M-BCR (p210). e14a2. Channel FAM™/ABY™



B) Detection of M-BCR (p210). e13a2. Channel FAM™/ABY™

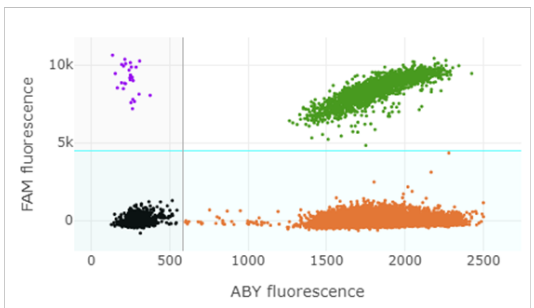


Figure 2. Two-dimensional (2D) comparative plots of M-BCR fusion transcript subtypes with the BCR-ABL1 Screening dPCR OncoKit. ***A)*** *control sample with M-BCR fusion transcript (subtype e14a2 or e14a3).* ***B)*** *control sample with M-BCR fusion transcript (subtype e13a2 or e13a3). The observed plots are obtained by selecting the specific fluorescence channel for M-BCR (FAM™) relative to ABL1 (ABY™).*

Table 5 summarizes the interpretation of possible results for BCR-ABL1 Screening dPCR OncoKit.

*Table 5. Possible outcomes when loading a cDNA sample, with + and – indicating the presence or absence of clusters. *The combined channels correspond to the green clusters, where the mutated transcript is amplified together with ABL1.*

Interpretation of results					
Sample	Channel FAM™	Channel VIC™	Channel JUN™	Channel ABY™	Combined channels
M-BCR positive	+	–	–	+	+
m-BCR positive	–	+	–	+	+
µ-BCR positive	–	–	+	+	+
Negative sample	–	–	–	+	–

Calculation of the frequency of the relevant fusion transcript in the sample

The analytical software analyzes the graphs obtained and reports the results in copies per microliter (cp/µL) based on the concentration of the targets in the dPCR reaction. This data is used to carry out *BCR-ABL1* transcript quantification expressed as a relative measure in relation to the *wild-type* gene, according to the following formula:

$$\%\ BCR\ ABL1 = \frac{BCR\ ABL1\ cp/\mu L}{ABL1\ cp/\mu L}\ x\ 100$$

Where:

- **BCR ABL1**: represents the copies/µL in FAM™, VIC™ or JUN™ reported by the analysis program.
- **ABL1**: represents the copies/µL for ABY™ reported by the analysis program for the *wild-type ABL1* gene.

For the assay to be considered valid, the user must ensure that the following conditions are met:

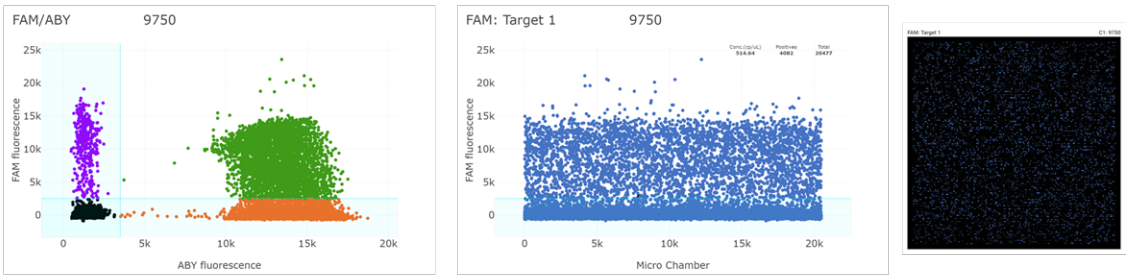
- There is no amplification signal from the PCR negative control in any of the configured channels.
- There is a signal in all channels (FAM™, VIC™, JUN™ y ABY™) in the reaction with the positive control.
- To reach the limit of detection of the kit (LoD 0.1%), it is necessary to obtain at least 1,100 cp/µL of *ABL1*.
- In samples with a high concentration of *BCR-ABL1* or *ABL1*, the BCR-ABL1 Screening dPCR OncoKit may generate INVALID results.

This condition can simultaneously affect:

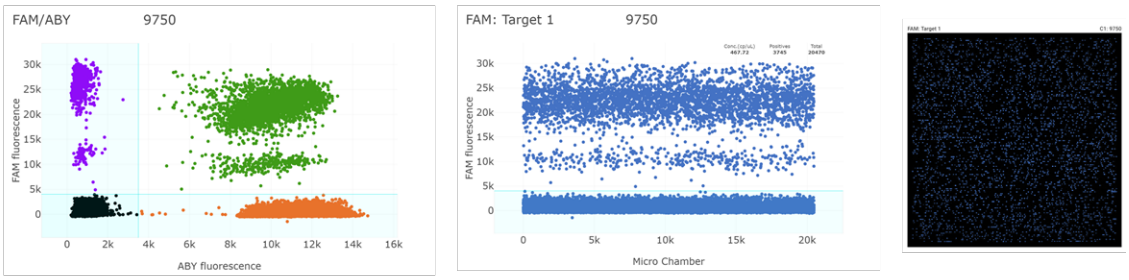
- Cluster separation. Combined clusters (green), where both the target and the endogenous control have been amplified, show significant noise, making it difficult to separate them from other clusters.
- *ABL1* transcript concentrations. Concentrations of ABL1 > 10,000 cp/µL (> 90,000 total copies) can saturate the system. It is recommended that ABL1 concentrations be kept in the range of 1,500–2,500 cp/µL to ensure good cluster separation and accurate quantification.

Figure 3 shows an example of a sample loaded at high concentrations. In such cases, it is recommended to dilute the sample. For more information, see section "10. Troubleshooting" of this manual.

A) Clinical sample positive for M-BCR-ABL1 diluted 1/2



B) Clinical sample positive for M-BCR-ABL1 diluted 1/4



C) Concentration and total copies per reaction of the detected targets at different dilutions

Clinical sample 9750	Dilution (1/2)		Dilution (1/4)	
Target (fluorophore)	M-BCR-ABL1 (FAM™)	*ABL1* (ABY™)	M-BCR-ABL1 (FAM™)	*ABL1* (ABY™)
Concentration	514.64 cp/µl	3,291.05 cp/µl	467.72 cp/µl	1,389.34 cp/µl
Total copies in 9 µ	4,631.76 cp	29,619.5 cp	4,209.48 cp	1,704.06 cp

Figure 3. Results from a clinical sample positive for the M-BCR-ABL1 fusion transcript at different cDNA dilutions (1/2 and 1/4 from the initial sample). ***A-B)*** *From left to right, 2D, 1D and fluorescence distribution along the microchamber are shown. At a loading dilution of 1/2 of the cDNA, abundant noise is observed, which prevents proper separation of the clusters. However, the microchamber does not show saturation* ***(A)****. At a loading dilution of 1/4 of the cDNA, the noise disappears, obtaining distinguishable clusters* ***(B)****.* ***C)*** *Concentration in copies/µL and total copies per reaction of the detected targets (M-BCR-ABL1 and ABL1) in the two dilutions tested.*

If other results than those shown in this section are obtained, please refer to section "10. Troubleshooting" of this manual.

10 Troubleshooting

Table 7 presents the expected results of a BCR-ABL1 Screening dPCR OncoKit assay for controls and samples, as well as invalid combinations, their potential causes, and solutions.

Table 7. Possible outcomes of the assay. Includes invalid results, their possible causes and solutions.

Control	Result		Cause
	FAM™ VIC™ JUN™	ABY™	
Positive control	+	+	Expected result
	–	–	Failed PCR amplification[1]
Sample	–	+	Expected result
	+	+	
	+	+	Saturated sample. Overlapping scatter plots[2]
	+	–	Invalid result[3]
	–	–	Failed PCR amplification[1] Sample amplification error[4]
PCR Negative control	–	–	Expected result
	+	+	Contamination of PCR with human DNA[5]
	+	–	
	–	+	

(1) Failed PCR amplification: check the amplification program and fluorescence capture settings. Amplification failures may result from technical issues related to PCR program settings or errors in preparing the reaction mixes.

(2) Saturation due to cDNA overload: samples with high concentrations of *BCR-ABL1* or *ABL1* transcripts may generate signals in the corresponding channels, but with significant noise (Figure 3A). In such cases, it is recommended to dilute the sample to obtain an ABL1 concentration of 1,500–2,500 cp/µL.

(3) Invalid result: no amplification of ABY™ (*ABL1* endogenous control). It is recommended to repeat the test.

(4) Sample amplification failure: the quality of the sample directly affects PCR performance. If the sample is highly degraded, lack of amplification may occur. On the other hand, if RT-PCR is performed with higher amounts of RNA than recommended, amplification failure may be due to saturation from excess cDNA. In such cases it is recommended to dilute the sample 1/10. If the clusters have not been satisfactorily distributed as shown in Figures 1 and 2 in the "Analysis of results" section, readjustments should be made to achieve concentrations of 1,500–2,500 cp/µL, depending on the obtained concentration.

(5) Contamination of the PCR process with human DNA: this may result from mishandling of the sample, use of contaminated reagents or environmental contamination. Thoroughly clean the laboratory where the PCR was prepared, as well as the equipment and materials used. If necessary, use new aliquots of PCR reagents. Prepare the PCR reaction containing the positive control as the final step, in order to avoid cross-contamination. In this case, it is recommended to repeat the test.

11 Limitations

11.1 | Analytical

The BCR-ABL1 Screening dPCR OncoKit is designed to detect M-BCR, m-BCR and µ-BCR fusion transcripts, but does not distinguish between subtypes. Its ability to detect other rare t(9;22)-derived fusion transcripts has not been evaluated. The assay does not detect micro deletions or point mutations.

It is not recommended for the diagnosis of CML or for determining treatment discontinuation or follow-up after treatment cessation.

Lower mRNA starting quantities than indicated during reverse transcription (see section "04. Conditions for sample collection, handling and preparation") may adversely affect the detection of *BCR-ABL1* transcripts present in very low numbers in the patient's blood.

In samples with a high concentration of *BCR-ABL1* or *ABL1*, BCR-ABL1 Screening dPCR OncoKit may generate INVALID results due to system saturation. In such cases, it is recommended to repeat the test with a diluted sample. For more information, see Table 7, section "10. Troubleshooting".

The assay can detect patients with *BCR-ABL1* transcripts even at very low levels (below the LoD of 0.1%). However, its accuracy is not guaranteed below the stated LoD. To achieve the specified accuracy, at least 1,100 cp/µL of *ABL1* must be obtained.

11.2 | Equipment

The BCR-ABL1 Screening dPCR OncoKit has been validated using the

- *QuantStudio™ Absolute Q™ Digital PCR System* (Thermo Fisher Scientific)

11.3 | Reagents

BCR-ABL1 Screening dPCR OncoKit has been validated using the reagents included in the kit and those recommended in section 6 of this manual (Equipment and materials required but not supplied).

This product is validated for blood collected in *PAXgene* tubes. Modifications to these procedures may affect test performance.

This product is validated with the *SuperScript™ VILO™ cDNA Synthesis Kit* (Thermo Fisher Scientific). Modifications to these procedures may affect test performance.

11.4 | Product stability

The optimum performance of this product is confirmed provided that the recommended storage conditions according to the optimum product date for each production batch are followed.

12 Performance characteristics

12.1 | Validation samples

The BCR-ABL1 Screening dPCR OncoKit is designed to analyze total genomic DNA (gDNA) extracted from peripheral blood or bone marrow samples.

For the analytical validation of the BCR-ABL1 Screening dPCR OncoKit synthetic DNA fragments were designed that contain the fusion transcripts of interest and the *ABL1* region used as an endogenous control. The kit was also validated with 14 positive clinical samples, including all 3 fusion transcripts. Additionally, 11 negative samples were used to determine the sensitivity and specificity of the kit.

12.2 | Limit of Blank (LoB) and Limit of Detection (LoD)

The LoB and LoD of the BCR-ABL1 Screening dPCR OncoKit were determined using the *QuantStudio™ Absolute Q™ Digital PCR System*.

To calculate the LoB, 11 negative samples were used in two different assays. The expected negative result was obtained in all cases, making it possible to establish a LoB with a 95% confidence interval (CI) of (0.02% ± 4x10-3). Considering the LoB obtained, the LoD was assessed in 11 replicates using a positive patient sample diluted to an allele frequency of 0.1%. The results obtained made it possible to establish a LoD of 0.1% with a 95% CI (0.08% ± 1.5x10-2).

12.3 | Specificity

The analytical specificity of the BCR-ABL1 Screening dPCR OncoKit was determined by analyzing 11 negative samples for any of the *BCR-ABL1* fusion transcripts. No *BCR-ABL1* signal was detected in any of the samples used above the established LoD.

12.4 | Reproducibility and repeatability

The repeatability and reproducibility performance parameters of the BCR-ABL1 Screening dPCR OncoKit were evaluated using synthetic DNA fragments with the fusion transcripts of interest at the established LoD of 0.1%.

To determine the repeatability of the assay, 5 replicates of each fusion transcript at 0.1% were loaded. Table 8 shows the repeatability of the assay for each fusion transcript with a 95% CI.

Table 8. Results of repeatability assessment for the BCR-ABL1 Screening dPCR OncoKit performed with samples containing the various BCR-ABL1 transcripts evaluated at the limit of detection. SD: standard deviation CI: confidence interval.

Replica	Expected result (%)	Result M-BCR (%)	Result m-BCR (%)	Result µ-BCR (%)
1	0.5%	0.13	0.14	0.19
2		0.19	0.11	0.10
3		0.14	0.17	0.13
4		0.15	0.10	0.10
5		0.16	0.11	0.16
6		0.07	0.13	0.13
7		0.11	0.13	0.14
8		0.12	0.12	0.11
9		0.17	0.09	0.13
10		0.21	0.14	0.14
Mean (%)		0.15	0.12	0.13
SD (%)		0.03	0.02	0.03
CI (95%)		$0.15 \pm 2x10^{-2}$	$0.12 \pm 1.5x10^{-2}$	$0.13 \pm 1.7x10^{-2}$

For reproducibility, 2 replicates per assay were performed on two different days (total of 20 determinations) in the following range of concentrations: 0.1%, 1%, 10%, 50%, and 100% for each of the fusion transcripts (see Table 9). The coefficient of variation (CV) never exceeded 25%, demonstrating the excellent accuracy of the BCR-ABL1 Screening dPCR OncoKit.

Table 9. Results of reproducibility assessment for the BCR-ABL1 Screening dPCR OncoKit performed with samples containing the various BCR-ABL1 transcripts evaluated at different frequencies. SD: standard deviation CV: coefficient of variation

BCR-ABL1 transcript	Expected frequency (%)	Mean Day 1	Mean Day 2	Mean	SD	CV (%)
M-BCR	0.10%	0.15	0.12	0.14	0.028	20.50
	1%	1.03	1.08	1.05	0.032	3.03
	10%	9.24	8.53	8.89	0.504	5.68
	50%	48.65	47.54	48.09	0.785	1.63
	100%	100.76	93.60	97.18	5.063	5.21
m-BCR	0.10%	0.11	0.13	0.12	0.011	9.54
	1%	0.86	0.82	0.84	0.030	3.544
	10%	8.64	8.94	8.79	0.215	2.45
	50%	48.80	48.33	48.56	0.333	0.69
	100%	104.13	98.97	101.55	3.649	3.59
µ-BCR	0.10%	0.12	0.09	0.11	0.024	22.65
	1%	1.40	1.32	1.36	0.053	3.92
	10%	8.64	8.94	8.79	0.215	2.45
	50%	62.66	62.16	62.41	0.356	0.57
	100%	104.13	107.35	105.74	2.274	2.15

12.5 | Sensitivity with clinical samples

To determine the sensitivity of the BCR-ABL1 Screening dPCR OncoKit 14 positive clinical samples were analyzed with the different *BCR-ABL1* fusion transcripts evaluated by qPCR using the CE-IVD screening and quantification kits from Health in Code, S.L. (**IMG-108**, **IMG-121** and **IMG-122**). The frequency of these variants ranged from 0.02% to 100%. The BCR-ABL1 Screening dPCR OncoKit detected variants present at a frequency below the LoD, however, taking into account the necessary correction with the LoB, these samples were determined to be negative. For the remaining samples, the expected variant was correctly identified, establishing a sensitivity of 100%.

Contact our Technical Department for any questions about the applications of this product or its protocols:

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